(54) Title: IMPROVEMENTS RELATING TO DOUBLE-STRANDED RNA INHIBITION

(57) Abstract: There are described ways of improving the efficiency of double stranded RNA inhibition as a method of inhibiting gene expression in nematode worms such as C. elegans. In particular, the invention relates to the finding that changes in the genetic background of C. elegans result in increased sensitivity to double-stranded RNA inhibition.

IMPROVEMENTS RELATING TO DOUBLE-STRANDED RNA INHIBITION

The present invention is concerned with ways of
improving the efficiency of double stranded RNA
inhibition as a method of inhibiting gene expression
in nematode worms such as *C. elegans*. In particular,
the invention relates to the finding that the
susceptibility of nematode worms such as *C. elegans* to
double stranded RNA inhibition is affected by changes
in the genetic background of the worms.

It has recently been described in Nature Vol 391,
pp.806-811, February 98, that introducing double
stranded RNA into a cell results in potent and
specific interference with expression of endogenous
genes in the cell, which interference is substantially
more effective than providing either RNA strand
individually as proposed in antisense technology. This
specific reduction of the activity
of the gene was also found to occur in the nematode
worm Caenorhabditis elegans (*C. elegans*) when the RNA
was introduced into the genome or body cavity of the
worm.

The present inventors have utilized the double
stranded RNA inhibition technique and applied it
further to devise novel and inventive methods of (i)
assigning functions to genes or DNA fragments which
have been sequenced in various projects, such as, for
example, the human genome project and which have yet
to be accorded a particular function, and (ii)
identifying DNA responsible for conferring a
particular phenotype. Such methods are described in
the applicant's co-pending application number WO
00/01846. Processes for introducing RNA into a living
cell, either *in vivo* or *ex vivo*, in order to inhibit
expression of a target gene in that cell are

additionally described in WO 99/32619.

Several different experimental approaches can be used to introduce double-stranded RNA into nematode worms in order to achieve RNA interference *in vivo*.
5   One of the most straightforward approaches is simple injection of double-stranded RNA into a body cavity. A more elegant solution is to feed the nematodes on food organisms, generally bacteria, which express a double stranded RNA of the appropriate sequence,
10 corresponding to a region of the target gene.

The present inventors have now determined that the phenomenon of RNA interference in nematodes following ingestion of food organisms capable of expressing double-stranded RNA is dependent both on
15 the nature of the food organism and on the genetic background of the nematodes themselves. These findings may be exploited to provided improved methods of double-stranded RNA inhibition.

Therefore, according to a first aspect of the
20 present invention there is provided a method of inhibiting expression of a target gene in a nematode worm comprising feeding to said nematode worm a food organism which is capable of producing a double-stranded RNA structure having a nucleotide sequence
25 substantially identical to a portion of said target gene following ingestion of the food organism by the nematode, wherein the nematode has a non wild-type genetic background selected to provide increased sensitivity to RNA interference as compared to wild
30 type.

Caenorhabditis elegans is the preferred nematode worm for use in the method of the invention although the method could be carried out with other nematodes and in particular with other microscopic nematodes,
35 preferably microscopic nematodes belonging to the genus *Caenorhabditis*. As used herein the term "microscopic" nematode encompasses nematodes of

approximately the same size as *C. elegans*, being of
the order 1mm long in the adult stage. Microscopic
nematodes of this approximate size can easily be grown
in the wells of a multi-well plate of the type
5   generally used in the art to perform mid- to high-
throughput screening.

It is an essential feature of this aspect of the
invention that the nematode has a non wild-type
genetic background which confers greater sensitivity
10   to RNA interference phenomena (abbreviated herein to
RNAi) as compared to the equivalent wild type
nematodes. As illustrated in the accompanying
examples, introduction of double-stranded RNA
(abbreviated herein to dsRNA) into a non wild-type
15   strain according to the invention results in greater
inhibition of expression of the target gene.
Depending on the nature of the target gene, this
greater level of inhibition may be detectable at the
phenotypic level as a more pronounced phenotype.

20   The nematode having non wild-type genetic
background may, advantageously, be a mutant strain.
Mutations which have the effect of increasing
susceptibility of the nematode to RNAi may, for
example, affect the stability of dsRNA or the kinetics
25   of dsRNA turnover within cells of the worm or the rate
of uptake of dsRNA synthesised by a food organism.
Suitable mutant strains include mutant strains
exhibiting knock-out or loss-of-function mutations in
one or more genes encoding proteins involved in RNA
30   synthesis, RNA degradation or the regulation of these
processes.

In one preferred embodiment, the nematode is a
mutant strain, more preferably a mutant *C. elegans*,
which exhibits reduced activity of one or more
35   nucleases compared to wild-type. Suitable strains
include mutant strains exhibiting knock-out or loss-
of-function mutations in one or more genes encoding

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nucleases, such as RNases. A particularly preferred
example is the *nuc-1* strain. This mutant *C.
elegans* strain is known *per se* in the art.

In a second preferred embodiment, the nematode is
5 a mutant strain, more preferably a mutant *C. elegans,*
which exhibits increased gut uptake compared to wild-
type. Particularly preferred examples of such strains
are the so-called *C. elegans* gun mutants described
herein. In a still further embodiment, the nematode
10 may be a transgenic worm comprising one or more
transgenes which increase gut uptake relative to wild-
type.

The term "increased gut uptake" as used herein is
taken to mean increased uptake of foreign particles
15 from the gut lumen and may encompass both increased
gut permeability and increased gut molecular transport
compared to wild-type *C. elegans.*

C. elegans feeds by taking in liquid containing
its food (e.g. bacteria). It then spits out the
20 liquid, crushes the food particles and internalises
them into the gut lumen. This process is performed by
the muscles of the pharynx. The process of taking up
liquid and subsequently spitting it out is called
pharyngeal pumping. Once the food particles have been
25 internalised via pharyngeal pumping their contents
must cross the gut itself in order to reach target
sites in the worm. There are multiple factors which
effect the uptake of compounds from the gut lumen to
the surrounding tissues. These include the action of
30 multi-drug resistance proteins, multi-drug resistance
related proteins and the P450 cytochromes as well as
other enzymes and mechanisms available for transport
of molecules through the gut wall.

C. elegans mutants which exhibit increased uptake
35 of foreign molecules through the gut may be obtained
from the *C. elegans* mutant collection at the *C.*

elegans Genetic Center, University of Minnesota, St
Paul, Minnesota, or may be generated by standard
methods. Such methods are described by Anderson in
Methods in Cell Biology, Vol 48, "*C. elegans*: Modern
5   biological analysis of an organism" Pages 31 to 58.
Several selection rounds of the PCR technique can be
performed to select a mutant worm with a deletion in a
desired gene. Alternatively, a population of worms
could be subjected to random mutagenesis and worms
10 exhibiting the desired characteristic of increased gut
uptake selected using a phenotypic screen, such as the
dye uptake method described herein.

As an alternative to mutation, transgenic worms
may be generated with the appropriate characteristics.
15 Methods of preparing transgenic worms are well known
in the art and are particularly described by Craig
Mello and Andrew Fire, Methods in Cell Biology, Vol
48, Ed. H.F. Epstein and D.C. Shakes, Academic Press,
pages 452-480.

20 Worms exhibiting the desired characteristics of
increased gut uptake can be identified using a test
devised by the inventors based on uptake of a marker
precursor molecule which is cleaved by the action of
enzymes present in the gut lumen to generate a marker
25 molecule which produces a detectable signal, such as
fluorescence. A suitable marker precursor molecule is
the fluorescent dye precursor BCECF-AM available from
Molecular Probes (Europe BV), Netherlands. This dye
only becomes fluorescent when cleaved by esterases and
30 maintained at a pH above 6. The pH of the gut lumen
is usually 5 or below. Thus, any BCECF-AM taken up
through the pharynx into the gut lumen is not
fluorescent until cleaved and the cleaved portion has
entered the cells surrounding the lumen which are at a
35 higher pH. Thus, this dye is able to quickly identify
mutant or otherwise modified worms which have
increased gut transport or permeability. There is a

gradual increase in fluorescence in the tissues
surrounding the gut while the gut lumen remains dark.
The fluorescence can be detected at an excitation
wavelength of 485 nm and an emission wavelength of 530
5   nm.

Specific examples of gun mutant strains isolated
using this procedure which may be used in the method
of the invention are strains bg77, bg84, bg85 and
bg86, although it is to be understood that the
10   invention is in no way limited to the use of these
specific strains. The *C. elegans* gun mutant strain
bg85 was deposited on 23 December 1999 at the BCCM/LMG
culture collection, Laboratorium Voor Microbiologie,
Universiteit Gent, K. L. Ledeganckstraat 35, B-9000,
15   Gent, Belgium under accession number LMBP 5334CB. The
phrase "the bg85 mutation" as used herein refers to
the specific mutation(s) present in the bg85 strain
which is/are responsible for conferring the gun
phenotype.
20   It is also within the scope of the invention to
use a non wild-type nematode strain, preferable a *C.
elegans* strain, having multiple mutations which affect
sensitivity to RNAi. A preferred type of multiple
mutant is one having at least one mutation which
25   results in reduced nuclease activity compared to wild
type and at least one mutation which results in
increased gut uptake compared to wild type. An
example of such a mutant is a *C. elegans* strain having
the *nuc-1* mutation and at least one further gun
30   mutation. As exemplified herein, double mutants
having the *nuc-1* mutation and a gun mutation exhibit
enhanced sensitivity to RNAi as compared to either
nuc-1 or gun single mutants.
For the avoidance of doubt, where particular
35   characteristics or properties of nematode worms are
described herein by relative terms such as "enhanced"

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or "increased" or "decreased" this should be taken to
mean enhanced, increased or decreased relative to
wild-type nematodes. In the case of *C. elegans,* wild-
type is defined as the N2 Bristol strain which is well

5   known to workers in the *C. elegans* field and has been
extremely well characterised (see Methods in Cell
Biology, Volume 48, Caenorhabditis elegans: Modern
biological analysis of an organism, ed. by Henry F.
Epstein and Diane C. Shakes, 1995 Academic Press; The

10   nematode Caenorhabditis elegans, ed. by William Wood
and the community of *C. elegans* researchers., 1988,
Cold Spring Harbor Laboratory Press; *C. elegans* II,
ed. by Donald L. Riddle, Thomas Blumenthal, Barbara J.
Meyer and James R. Priess, 1997, Cold Spring Harbor

15   Laboratory Press). The N2 strain can be obtained from
the *C. elegans* Genetic Center, University of
Minnesota, St Paul, Minnesota, USA.

The food organism for use in the above aspect of
the invention is preferably a bacterium such as, for

20   example, a strain of *E.coli*. It will, however, be
appreciated that any other type of food organism on
which nematodes feed and which is capable of producing
dsRNA could be used. The food organism may be
genetically modified to express a double-stranded RNA

25   of the appropriate sequence, as will be understood
with reference to the examples included herein. One
convenient way in which this may be achieved in a
bacterial food organism is by transforming the
bacterium with a vector comprising a promoter or

30   promoters positioned to drive transcription of a DNA
sequence to RNA capable of forming a double-stranded
structure. Examples of such vectors will be further
described below.

The actual step of feeding the food organism to

35   the nematode may be carried out according to
procedures known in the art, see WO 00/01846.

Typically the feeding of the food organisms to the
nematodes is performed on standard agar plates
commonly used for culturing *C. elegans* in the
laboratory. However, the step of feeding the food
5   organism to the nematodes may also be carried out in
liquid culture, for example in the wells of 96-well
microtitre assay plates.

The inventors have further observed that
variations in the food organism can result in enhanced
10   *in vivo* RNAi when the food organism is ingested by a
nematode worm.

Accordingly, in a further aspect the invention
provides a method of inhibiting expression of a target
gene in a nematode worm comprising feeding to said
15   nematode worm a food organism capable of producing a
double-stranded RNA structure having a nucleotide
sequence substantially identical to a portion of said
target gene following ingestion of the food organism
by the nematode, wherein the food organism carries a
20   modification selected to provide increased expression
or persistence of the doubled-stranded RNA compared to
a food organism which does not carry the modification.

The modification present in the food organism can
be any modification which results in increased
25   expression of the dsRNA or in increased persistence of
the dsRNA. Suitable modifications might include
mutations within the bacterial chromosome which affect
RNA stability and/or degradation or mutations which
have a direct effect on the rate of transcription. In
30   a preferred embodiment, the food organism is an RNAse
III minus *E. coli* strain, or any other RNAse negative
strain.

According to a still further aspect of the
invention there is provided a method of inhibiting
35   expression of a target gene in a nematode worm
comprising introduction of a DNA capable of producing
a double-stranded RNA structure having a nucleotide

sequence substantially identical to a portion of said
target gene in said nematode, wherein the nematode is
one which exhibits increased gut uptake compared to
wild type.

5   In addition to exhibiting increased sensitivity
to RNAi following feeding with food organisms capable
. of expressing a dsRNA, nematodes which exhibit
increased gut uptake as described herein also show
increased uptake of DNA molecules capable of producing
10   double-stranded RNA structures following ingestion
into a nematode.

           In a preferred embodiment, the DNA is in the form
of a vector comprising a promoter or promoters
orientated to relative to a sequence of DNA such that
15   they are capable of driving transcription of the said
DNA to make RNA capable of forming a double-stranded
structure upon binding of an appropriate RNA
polymerase to the promoter or promoters.

           Several different arrangements of promoters may
20   be used in such a vector. In a first arrangement a
DNA fragment corresponding to a region of the target
gene is flanked by two opposable polymerase-specific
promoters which are preferably identical.
Transcription from the opposable promoters produces
25   two complementary RNA strands which can anneal to form
an RNA duplex. The plasmid pGN1 described herein is
an example of a vector comprising two opposable T7
promoters flanking a multiple cloning site for
insertion of a DNA fragment of the appropriate
30   sequence, corresponding to a region of a target gene.
pGN8 is an example of a vector derived from pGN1
containing a fragment of the C. elegans unc-22 gene.
In an alternative arrangement, DNA fragments
corresponding to a region of the target gene may be
35   placed both in the sense and the antisense orientation
downstream of a single promoter. In this case, the
sense/antisense fragments are co-transcribed to

generate a single RNA strand which is self-
complementary and can therefore form an RNA duplex.

In both of the above arrangements, the
polymerase-specific T3, T7 and SP6 promoters, all of
5   which are well known in the art, are useful for
driving transcription of the RNA. Expression from
these promoters is dependent on expression of the
cognate polymerase. Advantageously, the nematode
itself may be adapted to express the appropriate
10   polymerase. Expression of the polymerase may be
general and constitutive, but could also be regulated
under a tissue-specific promoter, an inducible
promoter, a temporally regulated promoter or a
promoter having a combination of such characteristics.
15   Transgenic C. elegans strains harboring a transgene
encoding the desired polymerase under the control of
an appropriately-regulated promoter can be constructed
according to methods known per se in the art and
described, for example, by Craig Mello and Andrew Fire
20   in Methods in Cell Biology, Vol 48, Ed. H. F. Epstein
and D. C. Shakes, Academic Press, pp 452-480.

The advantage of adapting the nematode to express
the required polymerase is that it is possible to
control inhibition of expression of the target gene in
25   a tissue-specific and/or temporally specific manner by
placing expression of the polymerase under the control
of an appropriately regulated promoter.

Introduction of DNA into nematodes in accordance
with the method of the invention can be achieved using
30   a variety of techniques, for example by direct
injection into a body cavity or by soaking the worms
in a solution containing the DNA. If the DNA is in the
form of a vector as described herein, e.g. a plasmid
harboring a cloned DNA fragment between two flanking
35   T7 promoters, then dsRNA corresponding to this DNA
fragment will be formed in the nematode resulting in
down regulation of the corresponding gene. The

introduced DNA can form an extrachromosomal array,
which array might result in a more catalytic knock-out
or reduction of function phenotype. The DNA might also
become integrated into the genome of the nematode,
5   resulting in the same catalytic knock out or reduction
of function phenotype, but which is stably
transmittable.

In each aspect of the invention, the double-
stranded RNA structure may be formed by two separate
10   complementary RNA strands or a single self-
complementary strand, as described above. Inhibition
of target gene expression is sequence-specific in that
only nucleotide sequences corresponding to the duplex
region of the dsRNA structure are targeted for
15   inhibition.

It is preferred to use dsRNA comprising a
nucleotide sequence identical to a portion of the
target gene, although RNA sequences with minor
variations such as insertions, deletions and single
20   base substitutions may also be used and are effective
for inhibition. It will be readily apparent that 100%
sequence identity between the dsRNA and a portion of
the target gene is not absolutely required for
inhibition and the phrase "substantially identical" as
25   used herein is to be interpreted accordingly.
Generally sequences which are substantially identical
will share at least 90%, preferably at least 95% and
more preferably at least 98% nucleic acid sequence
identity. Sequence identity may be conveniently
30   calculated based on an optimal alignment, for example
using the BLAST program accessible at
WWW.ncbi.nlm.nih.gov.

The invention will be further understood with
reference to the following non-limiting Examples,
35   together with the accompanying Figures in which:

Figure 1 is a plasmid map of the vector pGN1

containing opposable T7 promoters flanking a multiple
cloning site and an ampicillin resistance marker.

Figure 2 is a plasmid map of the vector pGN8 (a
5   genomic fragment of the *C. elegans unc-22* gene cloned
in pGN1).

Figure 3 is a plasmid map of the vector pGN29
containing two T7 promoters and two T7 terminators
10   flanking *Bst*XI sites. This vector permits cloning of
DNA fragments linked to *Bst*XI adaptors.

Figure 4 *is a plasmid map of the vector pGN39
containing two* T7 promoters and two T7 terminators
15   flanking attR recombination sites (based on the
Gateway™ cloning system of Life Technologies, Inc).

Figure 5 is a plasmid map of the vector pGX22 (a
fragment of the *C. elegans* gene C04H5.6 cloned in
20   pGN29).

Figure 6 is a plasmid map of the vector pGX52 (a
fragment of the *C. elegans* gene K11D9.2b cloned in
pGN29).
25

Figure 7 is a plasmid map of the vector pGX104 (a
fragment of the *C. elegans* gene Y57G11C.15 cloned in
pGN29).

30   Figure 8 is a plasmid map of the vector pGZ8 (a
fragment of the *C. elegans* gene T25G3.2 cloned in
pGN39).

Figure 9 shows the results of an RNAi experiment in
35   which wild-type (N2) or *nuc-1* strain *C. elegans* in
liquid culture were fed with *E. coli* containing the

plasmid pGX22.

Figure 10 shows the results of an RNAi experiment in
which wild-type (N2) or *nuc-1* strain *C. elegans* in
5   liquid culture were fed with *E. coli* containing the
plasmid pGX52.

Figure 11 shows the results of an RNAi experiment in
which wild-type (N2) or *nuc-1* strain *C. elegans* in
10   liquid culture were fed with *E. coli* containing the
plasmid pGXGZ8.

Figure 12 shows the results of an RNAi experiment in
which wild-type (N2) or *nuc-1* strain *C. elegans* in
15   liquid culture were fed with *E. coli* containing the
plasmid pGX104

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Example 1
Influence of genetic background on the efficiency of
RNAi in *C. elegans*.

5   **Introduction**
Various different *C. elegans* strains were fed with
different bacteria, to test the possibility of RNAi by
feeding *C. elegans* with bacteria that produce dsRNA.
The possibility of DNA delivery and dsRNA delivery has
10   previously been envisaged by using different bacterial
strains. In this experiment the importance of the *C.
elegans* strain as receptor of the dsRNA is also shown.

For this experiment the following *E. coli* strains were
15   used:

1.   MC1061: F-*araD139* Δ*(ara-leu)7696 galE15 galK16*
      Δ*(lac)X74 rpsl (Str^r) hsdR2 (r$_k^-$ m$_k^+$) mcrA mcrB1*
      - regular host for various plasmids,
20   - Wertman et al., (1986) Gene 49:253-262,
      - Raleigh et al., (1989) in Current Protocols in
      Molecular Biology eds. Ausubel et al, Publishing
      associates and Wiley Interscience; New York. Unit
      1.4.
25

2.   B21(DE3): F- *ompT(lon) hsdS$_B$ (r$_B^-$,m$_B^-$;* an *E. coli* B
      strain) with DE3, a λ prophage carrying the T7
      RNA polymerase gene.
      - regular host for IPTG inducible T7 polymerase
30   expression,
      - Studier et al. (1990) Meth. Enzymol. 185:60-89

3.   HT115 (DE3): F- *mcrA mcrb* IN(rrnD-rrnE) 1 λ-
      rnc14::tr10 (DE3 lysogen: lacUV5
35   promoter-T7polymerase)
      - host for IPTG inducible T7 polymerase

expression,

- RNaseIII-,

- Fire A, Carnegie Institution, Baltimore, MD,
Pers. Comm.

For this experiment the following *C. elegans* strains
were used:

1.   C. elegans N2: regular WT laboratory strain

2.   *C. elegans nuc-1*(e1393): *C. elegans* strain with a
     reduced endonuclease activity (>95%); condensed
     chromatin persists after programmed cell death;
     ingested (bacterial) DNA in the intestinal lumen
     is not degraded. Several alleles are described:
     e1392 (strong allele: has been used for the
     experiments described below); n887 (resembles
     e1392) and n334 (weaker allele)
     - Stanfield et al. (1998) East Coast Worm
     meeting abstract 171,
     - Anonymous, Worm Breeder's Gazette 1(1):17b
     Hevelone et al. (1988) Biochem. Genet. 26:447-461
     - Ellis et al., Worm breeder's Gazette 7(2):44
     - Babu, Worm Breeder's gazette 1(2):10
     - Driscoll, (1996) Brain Pathol. 6:411-425
     - Ellis et al., (1991) Genetics 129:79-94

For this experiment the following plasmids were used:

pGN1: A vector encoding for ampicillin resistance,
harbouring a multiple cloning site between two
convergent T7 promoters.

pGN8: pGN1 containing a genomic fragment of *unc-22*.
Decreased unc-22 expression via RNAi results in a
"twitching" phenotype in *C. elegans*.

Experimental conditions

12-well micro-titer plates were filled with
approximately 2 ml of NGM agar per well (1 litre of
NGM agar: 15g Agar, 1g peptone, 3g NaCl, 1ml
cholesterol solution (5 mg/ml in EtOH), with sterile
addition after autoclaving of 9.5 ml 0.1M $CaCl_2$, 9.5
ml 0.1 ml $MgSO_4$, 25 ml 1M KH_2PO_4/K_2HPO_4 buffer pH 6 and
5 ml nystatin solution (dissolved 10 mg/ml in 1:1
EtOH:CH_3COONH_4 7.5 M).

The dried plates were spotted with approximately 50 µl
of an overnight culture of bacteria. When IPTG
induction was required, 50 µl of a 10 mM stock
solution of IPTG was dropped on top of the bacteria
lawn, and incubated at 37°C for approximately 4 hours.
Individual nematodes at the L4 growth stage were then
placed in single wells. In each well 4 nematodes, and
the plates were further incubated at 20°C for 6 days
to allow offspring to be formed. The F1 offspring of
the seeded nematodes were tested for the twitching
phenotype.

Results

Table 1:   Percentage of the offspring that show the
           twitching phenotype

MC1061	N2	nuc-1
pGN1	0%	0%
pGN1 + IPTG	0%	0%
pGN8	0%	0%
pGN8 + IPTG	0%	0%
BL21(DE3)		
pGN1	0%	0%
pGN1 + IPTG	0%	0%
pGN8	20% (+)	>90% (++)
pGN8 + IPTG	20% (+)	>90% (++±)
HT115(DE3)		
pGN1	0%	0%
pGN1 + IPTG	0%	0%
pGN8	50% (+±)	>90% (++)
pGN8 + IPTG	80% (++)	>90% (+++)

%: percentage twitchers

+: weak twitching

++: twitching

+++: strong twitching

Conclusions

The experiment with *E. coli* MC1061 shows that no
twitching could be observed in this experiment.
Neither the N2 nematodes nor the *nuc-1* nematodes
showed any twitchers. This is to be expected as *E.
coli* MC1061 does not produce any T7 RNA polymerase,
and hence the unc-22 fragment cloned in pGN8 is not

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expressed as dsRNA.

The experiment with *E. coli* strain BL21(DE3) and
nematode strain N2 shows expected results. BL21(DE3)
5   harbouring plasmid pGN1 does not result in any
twitching as the pGN1 vector is an empty vector. BL21
(DE3) harbouring PGN8 results in the expression of
unc-22 dsRNA. When this dsRNA is fed to the N2
nematode (indirectly by feeding with the bacteria that
10   produce the dsRNA), this results in a twitching
phenotype, indicating that the dsRNA is able to pass
the gut barrier and is able to perform its interfering
activity.

15   Surprisingly the RNAi effect of the unc-22 dsRNA was
even more pronounced in *C. elegans* strain *nuc-1* than
in the wild type N2 strain. Although one may expect
that the *nuc-1* mutation results in the non-degradation
or at least in a slower degradation of DNA, as the
20   NUC-1 protein is known to be involved in DNAse
activity, we clearly observe an enhancement of the
RNAi induced phenotype in *C. elegans* with a *nuc-1*
background. The *nuc-1* mutation has not been cloned
yet, but it has been described that the gene is
25   involved in nuclease activity, and more particularly
DNAse activity. If the NUC-1 protein is a nuclease, it
may also have activity on nuclease activity on dsRNA,
which would explain the enhanced RNAi phenotype. The
nuc-1 gene product may be a nuclease, or a regulator
30   of nuclease activity. As the mode of action of RNAi is
still not understood, it is also possible that the
NUC-1 protein is interfering in the mode of action of
RNAi. This would explain why a *nuc-1* mutant is more
sensitive to RNAi.
35

The experiment with the *E.coli* strain HT115 (DE3)

confirms the experiments with the BL21(DE3) strain.
The RNA interference observed with the unc-22 dsRNA is
even higher. In comparison with strain BL21(DE3) this
could be expected, as HT115(DE3) is a RNAse III minus

5   strain, and hence is expected to produce larger
amounts of dsRNA, resulting in more prominent RNAi.
This indicates further that the RNAi observed in this
experiment is the result of the dsRNA produced by the
bacteria fed to the *C. elegans*. Feeding *C. elegans*

10   *nuc-1* with HT115(DE3) harbouring pGN8 also results in
higher RNA interference phenotype than feeding the
same bacteria to *C. elegans* wild-type strain N2. Once
again this indicates that improved RNAi can realised
using a nuclease negative *C. elegans* and more

15   particularly with a with the *C. elegans nuc-1* (e1392)
strain.

Summary
RNA interference can be achieved in *C. elegans* by

20   feeding the worms with bacteria that produce dsRNA.
The efficiency of this RNA interference is dependent
both on the *E. coli* strain and on the genetic
background of the *C. elegans* strain. The higher the
level of dsRNA production in the *E. coli*, the more

25   RNAi is observed. This can be realised by using
efficient RNA expression systems such as T7 RNA
polymerase and RNAase negative strains, such as
RNaseIII minus stains. In this example the level of
dsRNA production varied: HT115(DE3)>BL21(DE3)>MC1061.

30
RNA interference is high in *C. elegans* strains that
are nuclease negative, or that are influenced in their
nuclease activity. This can be realised by using a
mutant strain such as *C. elegans nuc-1*.

35   In this example the sensitivity to RNAi varied:
C. elegans nuc-1 >> C. elegans N2

Example 2

Improved RNAi by feeding dsRNA producing bacteria in
selected *C. elegans* strains-Comparison of the *nuc-1*
strain with several mutants which show improved gut
5   uptake (designated herein 'gun' mutants). Strains
bg77, bg78, bg83, bg84, bg85, bg86, bg87, bg88 and
bg89 are typical gun mutant *C. elegans* strains
isolated using selection for increased gut uptake (gun
phenotype) with the marker dye BCECF-AM.
10

Experimental conditions:
- 12-well micro-titer plates were filled with
approximately 2ml of NGM agar (containing 1ml/l
of ampicillin (100µg/ml) and 5 ml of 100mM stock
15   IPTG) per well
- the dried plates were spotted with 25µl of an
overnight culture of bacteria (BL21DE3/HT115DE3)
containing the plasmids pGN1 (T7prom-T7prom) or
pGN8 (T7prom-unc-22-T7prom)
20   - individual nematodes at the L4 growth stage were
then placed in single wells, one nematode per
well
- the plates were incubated at 20°C for 6 days to
allow offspring to be formed
25   - the adult F1 offspring of the seeded nematodes
were tested for the twitching phenotype

Results:

Table 2:

20°C/6d	pGN1 HT115DE3	pGN8 BL2DE3	pGN8 HT115DE3	
N2	0	1	1	
nuc-1	0	1-2	3	
bg77	0	1-2	3	
bg78	0	1	1-2	
bg83	0	1	1	
bg84	0	1-2	8	
bg85	0	1	2-3	
bg86	0	1	2	
bg87	0	1	1	
bg88	0	1	1	
bg89	0	1	1	

figure legend:

    0 = no twitching

    1 = no to weak phenotype

    2 = clear phenotype

    3 = strong phenotype

Conclusions

- bacterial strain HT115(DE3) shows a better RNAi sensitivity than bacterial strain BL21(DE3)
- the *nuc-1 C. elegans* strain is a better strain than the Wild-type N2 strain for RNAi sensitivity
- various gun mutants (improved gut uptake mutants) and more particularly the gun mutant strains bg77, bg84, bg85, bg86 show improved sensitivity to RNAi compared to Wild-type.

A double mutant *C. elegans* strain (nuc-1/gun) shows
even greater sensitivity to RNAi compared to
wild-type:

5   Double mutants were constructed to test the prediction
       that gun/nuc mutants would even show more enhanced
       RNAi sensitivity. As an example, the crossing
       strategy with gun strain bg85 is shown, similar
       crosses can be conducted with other gun strains, such

10   as bg77, bg84 and bg86.

       P0 cross: gun(bg85) x WT males

       F1 cross: nuc-1 x gun(bg85)/+ males

15

       F2 cross: nuc-1 x gun(bg85)/+; nuc-1/0 males (50%)
                 nuc-1 x +/+; nuc-1/0 males (50%)

       F3 single: gun(bg85)/+; nuc-1 hermaphrodites (25%)
20   +/+; nuc-1 hermaphrodites (75%)

       F4 single: gun(bg85); nuc-1 (1/4 of every 4th
                       plate high staining with BCECF)

25   F5 retest: gun(bg85); nuc-1 (100% progeny of F4
                       singled high staining with BCECF)

       To select for the gun phenotype, the fluorescence
       precursor BCECF-AM is used (obtainable from Molecular
30   probes). The precursor BCECF-AM is cleaved by
       esterases present in the gut of the worm to generate
       the dye BCECF which is fluorescent at pH values above
       6. This allows selection for worms that have a gun
       phenotype. BCECF-AM is taken up through the pharynx
35   into the gut lumen and is not fluorescent until it has
       been cleaved, and the BCECF portion has entered the

cells surrounding the lumen. Wild-type worms will
show slower or no increase in BCECF fluorescence.

5   **Example 3**
Improved RNAi feeding in liquid culture using *nuc-
1*(e1393) *C. elegans*.

Introduction
10   N2 and nuc-1 *C.elegans* strains were fed with bacteria
producing dsRNAs that give lethal phenotypes via RNAi.
For this example RNAi was performed in liquid culture
instead of on agar plates. We show here for a number
of genes that the RNAi effect is more penetrant using
15   the nuc-1 strain than the N2 strain, and that RNAi can
be performed in liquid.

For this experiment the following *E.coli* strains were
used:
20

1.   HT115 (DE3): F- *mcrA mcrb IN*(rrnD-rrnE) 1 λ-
      rnc14::tr10 (DE3 lysogen: lacUV5 promoter -T7
      polymerase)
      - host for IPTG inducible T7 polymerase expression
25   - RNaseIII⁻
      - Fire A, Carnegie Institution, Baltimore, MD,
        Pers. Comm.

For this experiment, following *C. elegans* strains were
30   used:

1. *C. elegans* N2: regular WT laboratory strain

2.   *C. elegans nuc-1*(e1393): *C. elegans* strain with a
35   reduced endonuclease activity (>95%); condensed
           chromatin persists after programmed cell death;

ingested (bacterial) DNA in the intestinal lumen
is not degraded. Several alleles are described:
e1392 (strong allele: has been used for the
experiments described below); n887 (resembles
5   e1392) and n334 (weaker allele)
        - Stanfield et al. (1998) East Coast Worm meeting
          abstract 171
        - Anonymous, Worm Breeder's Gazette 1(1):17b
        - Hevelone et al. (1988) Biochem. Genet. 26:447-461
10   - Ellis et al., Worm breeder's Gazette 7(2):44
        - Babu, Worm Breeder's gazette 1(2):10
        - Driscoll, (1996) Brain Pathol. 6:411-425
        - Ellis et al., (1991) Genetics 129:79-94

15

For this experiment, the following plasmids that all
give lethal phenotypes in *C. elegans* via RNAi were
used:

20   pGX22: a vector encoding ampicillin resistance,
        containing a genomic fragment of cosmid C04H5.6
        corresponding to a member of the RNA helicase family.

        pGX52: a vector encoding ampicillin resistance,
25   containing a genomic fragment of cosmid K11D9.2b
        corresponding to sarco/endoplasmic Ca2$^+$ ATPase also
        known as SERCA.

        pGZ18: a vector encoding ampicillin resistance,
30   containing a genomic fragment of cosmid T25G3.2
        corresponding to a chitin like synthase gene.

        pGX104: a vector encoding ampicillin resistance,
        containing a genomic fragment of cosmid Y57G11C.15
35   corresponding to sec-61, a transport protein.

Experimental conditions

- 1 ml overnight cultures of HT115 (DE3) bacteria
  containing the plasmids pGX22, pGX52, pGZ18 or
  pGX104 respectively were pelleted and resuspended
5   in S-complete medium, containing 1ml/l of
  ampicillin (100 µg/ml) and 1ml/l of 1000mM IPTG.

- 10 µl of this bacterial solution was transferred
  to a 96-well microtiter plate already filled with
10   100 µl S-complete containing 1ml/l of ampicillin
  (100 µg/ml) and 1ml/l of 1000mM IPTG.

- 3 nematodes at the L1 growth stage of N2 and
  nuc-1 strain were then placed in single wells, 3
15   L1's per well. Per experimental set up, 16 wells
  were used (n=16).

- the plates were incubated at 25°C for 5 days to
  allow offspring to be formed.
20

- the plates were visually checked and the
  following phenotypes could be scored per
  individual well:

25 **no effect**: L1's developed to adults and gave normal
offspring.

no F1 offspring: L1's developed to adults and gave no
offspring.
30

acute lethal: original L1 did not mature and died.

Results
35 The results of this experiment are illustrated
graphically in Figures 9 to 12. Data are expressed as

- 26 -

a percentage of the total (n=16) on the y-axis for
both N2 and *nuc-1* strains.

Conclusions

5   The following genes were tested in this liquid RNAi
     assay:

         - C04H5.6: an RNA helicase. RNAi of this gene
           interferes with the generation of offspring.
10   - SERCA: a sarco/endoplasmic Ca^{2+} ATPase. A strong
           RNAi phenotype causes an acute lethal phenotype.
           A less penetrant RNAi effect results in loss of
           offspring.
         - T25G3.2: a chitin like synthase gene. RNAi of
15   this gene causes dead eggs.
         - sec-61: a transport protein. A strong RNAi
           phenotype causes an acute lethal phenotype. A
           less penetrant RNAi effect results in loss of
           offspring.
20   - RNAi can be performed under liquid conditions.

     As in the previous examples this set of experiments
     shows that the nuc-1 *C. elegans* strain is more
     sensitive to RNAi than the wild-type N2 strain. This
25   is most clear for less penetrant phenotypes such as
     SERCA and chitin synthase. For strong RNAi phenotypes
     like the helicase and Sec-61 the difference between
     the N2 wild-type strain and the nuc-1 stain is less
     pronounced.
30

Example 4

Cloning of pGX22, pGX52, pGZ18 and pGX104 for RNAi
A set of primers for each gene was designed on the
basis of sequence data available in the publicly
5   accessible *C. elegans* sequence database (Acedb).

The cosmid names relate to:

1. **C04H5.6**=member of RNA helicase
10   2. **K11D9.2b**=SERCA
3. **Y57G11C.15**=transport protein sec-61
4. **T25G3.2**=chitin synthase like

The following primer sequences were designed:
15

1. **C04H5.6F**   5'-TGCTCAGAGAGTTTCTCAACGAACC-3'
C04H5.6R   5'-CAATGTTAGTTGCTAGGACCACCTG-3'

2. **K11D9.2bF** 5'-CAGCCGATCTCCGTCTTGTG-3'
20   **K11D9.2bR** 5'-CCGAGGGCAAGACAACGAAG-3'

3. **Y57G11C.15F** 5'-ACCGTGGTACTCTTATGGAGCTCG-3'
Y57G11C.15R 5'-TGCAGTGGATTGGGTCTTCG-3'

25   4. **T25G3.2F**
5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCCAAGTACATGTCGATTGCG-3'

T25G3.2R
5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTGGAGAAGCATTCCGAGAGTTTG-3'
30

PCR was performed on genomic DNA of N2 strain *C.
elegans* to give PCR products of the following sizes:

35   1326bp for C04H5.6
1213bp for K11D9.2b

1024bp for Y57G11C.15
1115bp for T25G3.2

5   The PCR fragments of C04H5.6, K11D9.2b and Y57G11C.15
were linked to *Bst*XI adaptors (Invitrogen) and then
cloned into the pGN29 vector cut with *Bst*XI. pGN29
contains two T7 promoters and two T7 terminators
flanking a cloning site which is adapted for
facilitated cloning of PCR fragments, comprising a
10   stuffer DNA flanked by two *Bst*XI sites (see schematic
Figure 3). The resulting plasmids were designated
pGX22 (C04H5.6), pGX52 (K11D9.2b) and pGX104
(Y57G11C.15).

15   The PCR fragment of T25G3.2 was cloned into pGN39 via
recombination sites based on the GATEWAY™ cloning
system (Life Technologies, Inc). pGN39 contains two
T7 promoters and two T7 terminators flanking a cloning
site which facilitates "High Throughput" cloning based
20   on homologous recombination rather than restriction
enzyme digestion and ligation. As shown schematically
in Figure 4, the cloning site comprises *att*R1 and
*att*R2 recombination sites from bacteriophage lambda
flanking a gene which is lethal to *E. coli*, in this
25   case the *ccd*B gene. This cloning site is derived from
the Gateway™ cloning system commercially available
from Life Technologies, Inc. The Gateway™ cloning
system has been extensively described by Hartley et
al. in WO 96/40724 (PCT/US96/10082).

30

Example 5

Selecting *C. elegans* mutations for increased gut
uptake (gun) using marker dye BCECF-AM and *unc-31* as
background.

The screen was performed in *unc-31(e928)* mutant
background, to ensure high amounts of dye in the gut
lumen, since *unc-31* mutations show constitutive
pharyngeal pumping. The dye (BCECF-AM: 2',7' bis (2-
carboxyethyl)-5-(and-6)-carboxyfluorescein,
acetoxymethylester), obtained from Molecular Probes,
is cleaved by intracellular esterases. Fluorescence
accumulates in the gut cells upon passage through the
apical gut membrane.

<u>Mutagenesis</u>

Day 1:   *unc-31* L4 staged worms were mutagenised with
         EMS (final concentration 50 mM) for 4 hours
Day 2:   P0 was divided over several large agar
         plates
Day 6:   F1's were collected and dropped on large
         plates. The number of eggs the F1's layed
         were checked every hour and de F1's were
         removed when 10-20 eggs per F1 were counted
Day 10: F2 adults were collected and screened with
         BCECF-AM. Mutations with increased staining
         of the gut cells after 15-30 minutes
         exposure to the dye were selected and
         singled on small agar plates.

About 50 initial positives gave progeny which was
retested with BCECF-AM (2x) and leucine CMB (1x)
9 of the 50 strains were kept (2 strains : 3 times
positive, 7 other strains : twice positive)

Table 3:   Isolation of mutations for increased
            staining with BCECF-AM

Total P0	Total F1	Total F2	screened chromosomes	number of strains isolated
(counted)	(estimated)	(calculated)	(estimated)	(counted)
2251	55618	222472	100000	9

Outcrossing, backcrossing and double construction

1.   backcrossing *unc-31; gun* --> *unc-31; gun*
- *unc-31; gun* x WT males
- singled 2x5 WT hermaphrodites F1s (*unc-31/+;gun/+*)
- singled 50 WT hermaphrodites F2s (1/4 homozygous)
- select strains segregating 1/4 unc
- stain unc strains with BCECF-AM
- from positive strains pick unc homozygous
- retest 100 % unc strains with BCECF-AM
- kept 1 strain (backcrossed)

2.   *unc-31* background was crossed out-->+; *gun*
- *unc-31; gun* x WT males
- singled 2x5 WT hermaphrodites F1s (*unc-31/+;gun/+*)
- singled 50 WT hermaphrodites F2s (1/4 homozygous)
- select strains which did not segregate unc F3s
anymore
- stain non unc strains with BCECF-AM
- 7 positive strains were retested with BCECF-AM and
finally 1 was selected and kept (outcrossed)

3.   +; *gun* (1x outcrossed) were 2 times backcrossed--
>+; *gun* (3x backcrossed)
- *gun* x WT males
- WT hermaphrodites x F1 males (*gun/+*)
- singled 10 WT hermaphrodites F2s (½ heterozygous)
- singled 50 WT hermaphrodites F3s (1/8 homozygous)

- stain strains with BCECF-AM- retested positives with
BCECF-AM and finally 1 was selected and kept

4.   *gun* (3x backcrossed) were crossed with *nuc-1*(X)
mutant--> *gun; nuc-1*
- *gun* x WT males
- *nuc-1* x *gun/+* males
- *nuc-1* x *gun/+; nuc-1/0* or *+/+; nuc-1/0* males
- singled 10 WT hermaphrodite progeny (*nuc-1*
homozygous, ½ heterozygous *gun*)
- singled 40 WT hermaphrodite progeny (1/8 homozygous
gun)
- stain strains with BCECF-AM
- retested positives with BCECF-AM and finally 1 was
selected and kept

Table 6: Strains derived from gun mutations

gun	unc-31; gun		unc-31; gun		+; gun			gun; nuc-1
	original isolate		backcrossed (1x)		outcrossed (1x)		3x b.c.	from 3x b.c.
allele number	Isolation number	strain number	Isolation number	strain number	Isolation number	strain number	strain number	strain number
bg77	31.4	UG 510	31.4.46.1	UG 556	31.4.34	UG 563	UG 674	UG 777
bg78	37.5	UG 511	37.5.46.4	UG 557	37.5.15	UG 564	UG 675	–
bg83	10.2	UG 543	10.2.11	UG 600	10.2.21	UG 586	UG 676	–
bg84	7.2	UG 544	7.2.10	UG 601	7.2.15	UG 589	UG 677	UG 774
bg85	11.5	UG 545	11.5.29.2	UG 602	2x b.c.	UG 717		UG 775
bg86	42.1	UG 546	42.1.4.5	UG 603	42.1.18	UG 587	UG 678	UG 776
bg87	7.1	UG 547	7.1.8.3	UG 604	7.1.22	UG 585	UG 679	–
bg88	5.3	UG 548	5.3.9	UG 605	5.3.18	UG 584	UG 680	–
bg89	23.4	UG 549	23.4.13.5	UG 606	23.4.3	UG 588	UG 671	–

SEQUENCE LISTING:

        SEQ ID NO: 1   complete sequence of pGN1

5   SEQ ID NO: 2   complete sequence of pGN8

        SEQ ID NO: 3   complete sequence of pGN29

        SEQ ID NO: 4   complete sequence of pGN39
10
        SEQ ID NO: 5   complete sequence of pGX22

        SEQ ID NO: 6   complete sequence of pGX52

15   SEQ ID NO: 7   complete sequence of pGX104

        SEQ ID NO: 8   complete sequence of pGZ8

        SEQ ID NO: 9   primer C04H5.6F
20
        SEQ ID NO: 10 primer C04H5.6R

        SEQ ID NO: 11 primer K11D9.2bF

25   SEQ ID NO: 12 primer K11D9.2bR

        SEQ ID NO: 13 primer Y57G11C.15F

        SEQ ID NO: 14 primer Y57G11C.15R
30
        SEQ ID NO: 15 primer T25G3.2F

        SEQ ID NO: 16 primer T25G3.2R

Claims:

1.   A method of inhibiting expression of a
target gene in a nematode worm comprising feeding to
said nematode worm a food organism which is capable of
producing a double-stranded RNA structure having a
nucleotide sequence substantially identical to a
portion of said target gene following ingestion of the
food organism by the nematode, wherein the nematode
has a non wild-type genetic background selected to
provide increased sensitivity to RNA interference as
compared to wild type.

2.   A method as claimed in claim 1 wherein the
nematode is a microscopic nematode.

3.   A method as claimed in claim 2 wherein the
nematode is from the genus *Caenorhabditis*.

4.   A method as claimed in claim 3 wherein the
nematode is *C. elegans*.

5.   A method as claimed in any one of claims 1
to 4 wherein the nematode has a mutant genetic
background.

6.   A method as claimed in claim 5 wherein the
nematode is a mutant strain which exhibits reduced
activity of one or more nucleases compared to wild
type.

7.   A method as claimed in claim 6 wherein the
nematode is *C. elegans* strain *nuc-1*.

8.   A method as claimed in claim 5 wherein the
nematode is a mutant strain which exhibits increased

gut uptake compared to wild type.

9.   A method as claimed in claim 8 wherein the nematode is mutant *C. elegans* strain bg85.

10.   A method as claimed in claim 5 wherein the nematode is a mutant strain having at least one mutation which results in reduced nuclease activity compared to wild type and at least one mutation which results in increased gut uptake compared to wild type.

11.   A method as claimed in claim 10 wherein the nematode is a mutant *C. elegans* strain having the *nuc-1* mutation and the bg85 mutation.

12.   A method as claimed in any one of the preceding claims wherein the food organism has been engineered to express a double-stranded RNA.

13.   A method as claimed in any one of the preceding claims wherein the food organism is a bacterium.

14.   A method as claimed in claim 13 wherein the food organism is *E. coli*.

15.   A method as claimed in any one of the preceding claims wherein the food organism has been genetically modified to express a double-stranded RNA having a nucleotide sequence substantially identical to a portion of said target gene.

16.   A method as claimed in claim 15 wherein the food organism contains a DNA vector, the vector comprising a promoter or promoters orientated relative to a DNA sequence such that they are capable of

initiating transcription of said DNA sequence to RNA
capable of forming a double-stranded structure upon
binding of an appropriate RNA polymerase to said
promoter or promoters.

5

       17.   A method as claimed in claim 25 wherein the
vector comprises two promoters flanking the DNA
sequence.

10   18.   A method as claimed in claim 26 wherein the
two promoters are identical.

       19.   A method as claimed in claim 25 wherein the
vector comprises a single promoter and further
15   comprises said DNA sequence in a sense and an
antisense orientation relative to said promoter.

       20.   A method as claimed in any one of claims 16
to 20 wherein the nematode or the food organism is
20   adapted to express an RNA polymerase capable of
initiating transcription from said promoter or
promoters.

       21.   A method as claimed in any one of claims 16
25   to 20 wherein the RNA polymerase is T7, T3 or SP6
polymerase.

       22.   A method as claimed in any one of claims 1
to 21 wherein the step of feeding said food organism
30   to said nematode worm is carried out in liquid
culture.

       23.   A method of inhibiting expression of a
target gene in a nematode worm comprising feeding to
35   said nematode worm a food organism capable of
producing a double-stranded RNA structure having a
nucleotide sequence substantially identical to a

portion of said target gene following ingestion of the
food organism by the nematode, wherein the food
organism carries a modification selected to provide
increased expression or persistence of the doubled-
5   stranded RNA compared to a food organism which does
not carry the modification.

      24.   A method as claimed in claim 23 wherein the
food organism is a bacterium.
10

      25.   A method as claimed in claim 24 wherein the
bacterium is an *E. coli* strain.

      26.   A method as claimed in claim 25 wherein the
15   *E. coli* strain is an RNAse III minus strain or any
other RNAse negative strain.

      27.   A method as claimed in any one of claims 23
to 26 wherein the step of feeding said food organism
20   to said nematode worm is carried out in liquid
culture.

      28.   A method of inhibiting expression of a
target gene in a nematode worm comprising introduction
25   of a DNA capable of producing a double-stranded RNA
structure having a nucleotide sequence substantially
identical to a portion of said target gene in said
nematode, wherein the nematode is one which exhibits
increased gut uptake compared to wild type.
30

      29.   A method as claimed in claim 28 wherein the
nematode is a microscopic nematode.

      30.   A method as claimed in claim 29 wherein the
35   nematode is from the genus *Caenorhabditis*.

      31.   A method as claimed in claim 30 wherein the

nematode is *C. elegans.*

32. A method as claimed in any one of claims 28 to 31 wherein the nematode has a mutant genetic
5 background.

33. A method as claimed in claim 32 wherein the nematode is mutant *C. elegans* strain bg85.

10 34. A method as claimed in any one of claims 28 to 33 wherein the DNA capable of producing a double-stranded RNA structure is a vector comprising a promoter or promoters orientated relative to a DNA sequence such that they are capable of initiating
15 transcription of said DNA sequence to RNA capable of forming a double-stranded structure upon binding of an appropriate RNA polymerase to said promoter or promoters.

20 35. A method as claimed in claim 34 wherein the vector comprises two promoters flanking the DNA sequence.

36. A method as claimed in claim 35 wherein the
25 two promoters are identical.

37. A method as claimed in claim 34 wherein the vector comprises a single promoter and further comprises said DNA sequence in a sense and an
30 antisense orientation relative to said promoter.

38. A method as claimed in any one of claims 34 to 37 wherein the nematode is adapted to express an RNA polymerase capable of initiating transcription
35 from said promoter or promoters.

39. A method as claimed in any one of claims 34

to 38 wherein the RNA polymerase is T7, T3 or SP6
polymerase.

FIG. 1.

FIG. 2.

FIG. 3.

PHAGE F1

LAC OPERON

T7 P

T7-terminator

Bst XI (818)

polylinker & stuffer

Bst XI (1174)

T7-terminator

SP6 P

T7

LAC OPERON

APr

pGN29
3774 bp
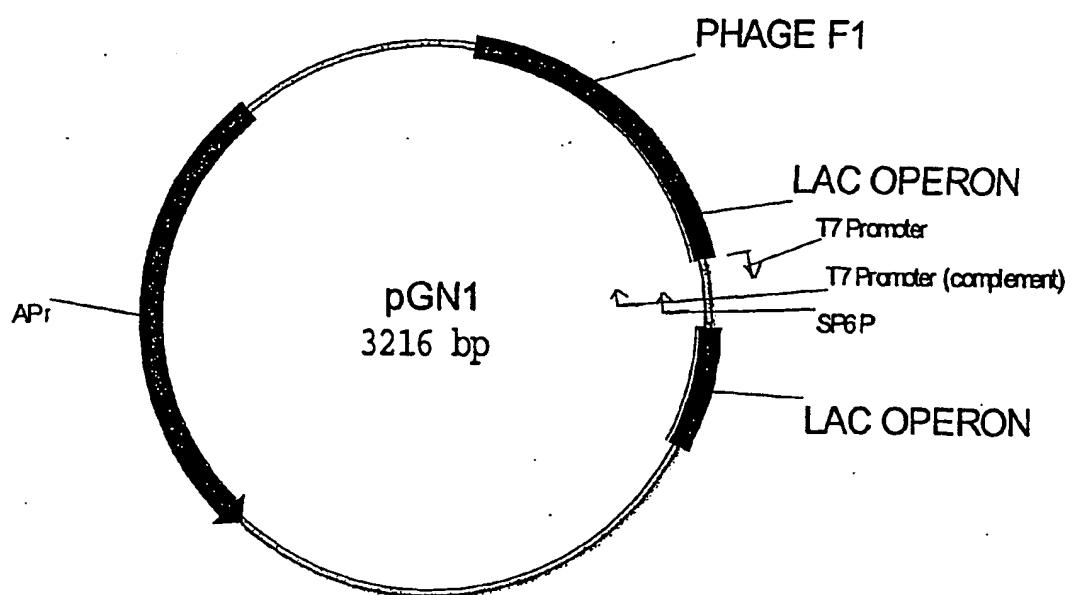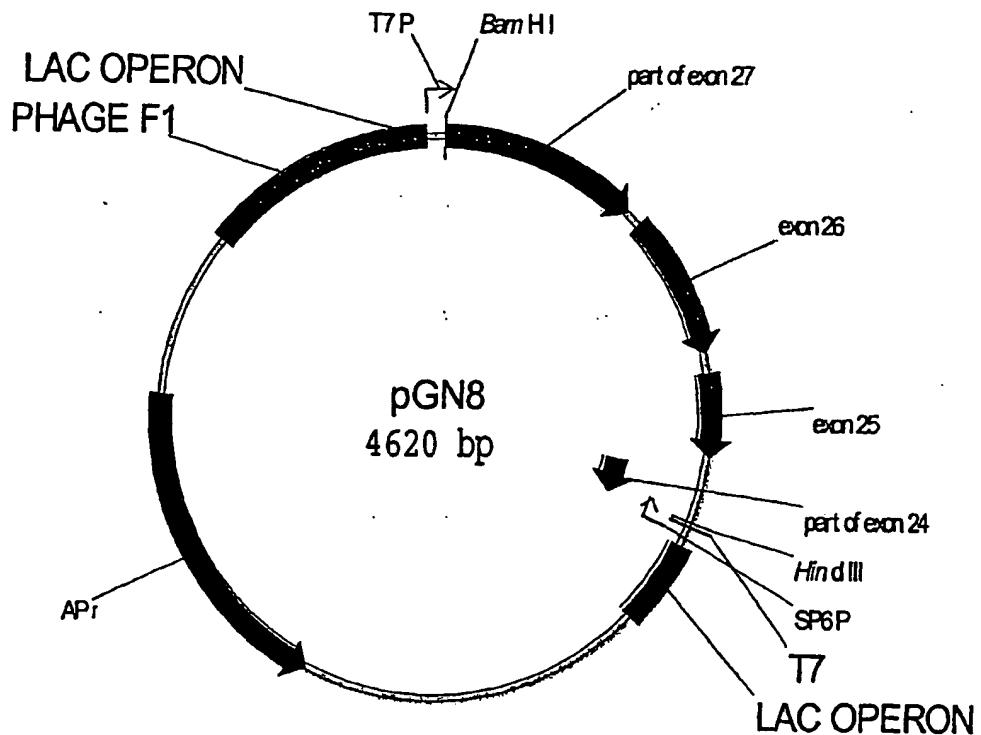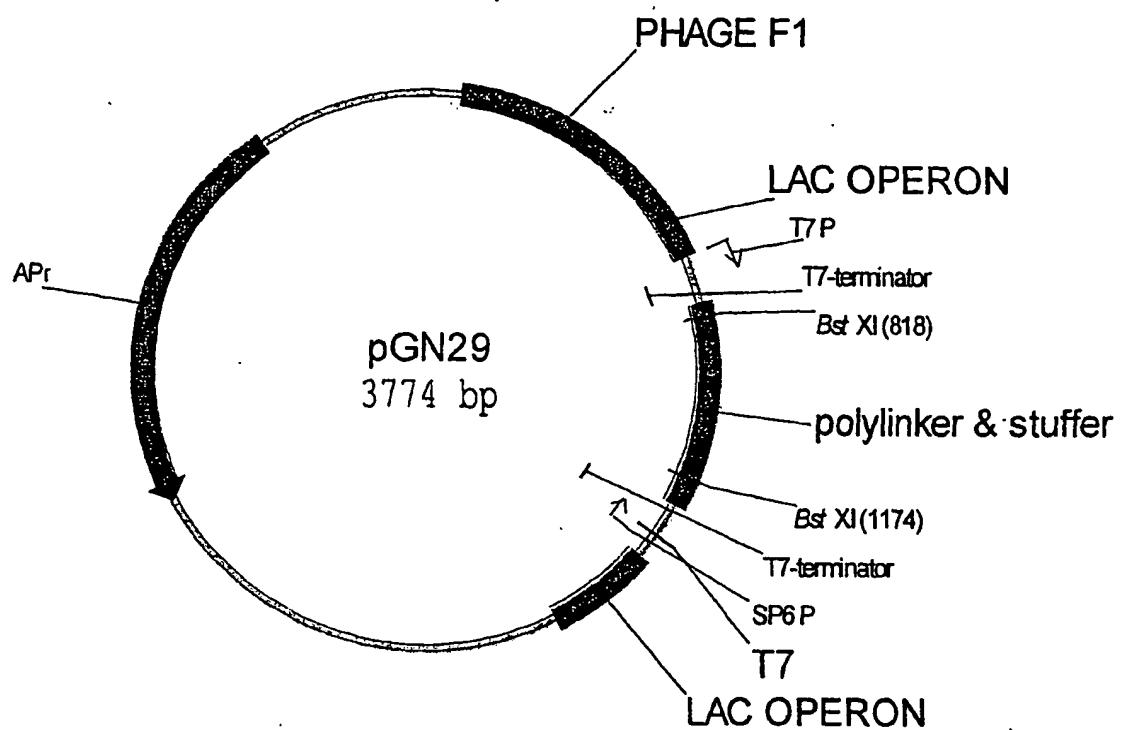
FIG. 4.

FIG. 5.

FIG. 6.

FIG. 7.

FIG. 8.

FIG. 9.

Helicase, pGX22

Legend: no effect / no F1 offspring

RNAi liquid screen

FIG. 10.

SERCA, pGX52

Legend: no effect / no F1 offspring / acute lethal

RNAi liquid screen

FIG. 11.

Chitine synthase, pGZ18

RNAi liquid screen

- ☐ no effect
- ▦ dead eggs

FIG. 12.

Sec-61, pGX104

RNAi liquid screen

- ☐ no F1 offspring
- ☐ acute lethal
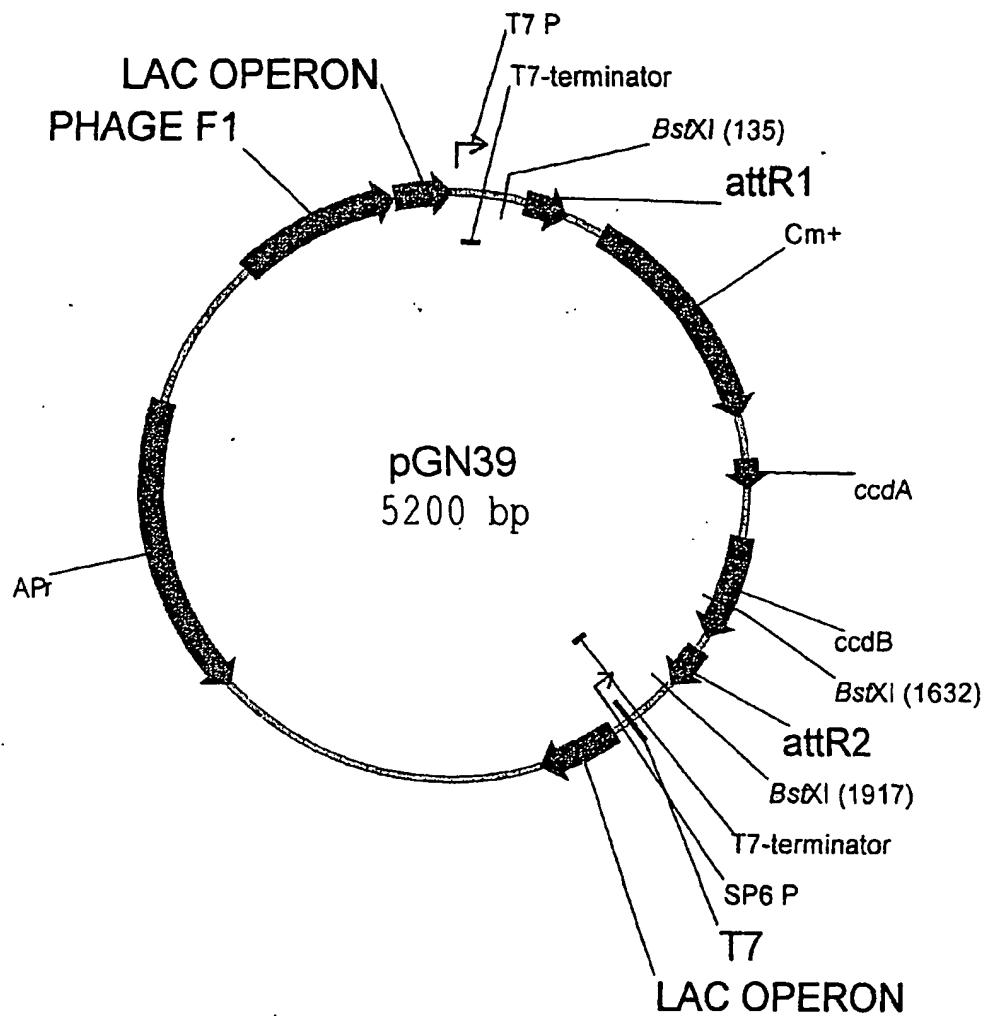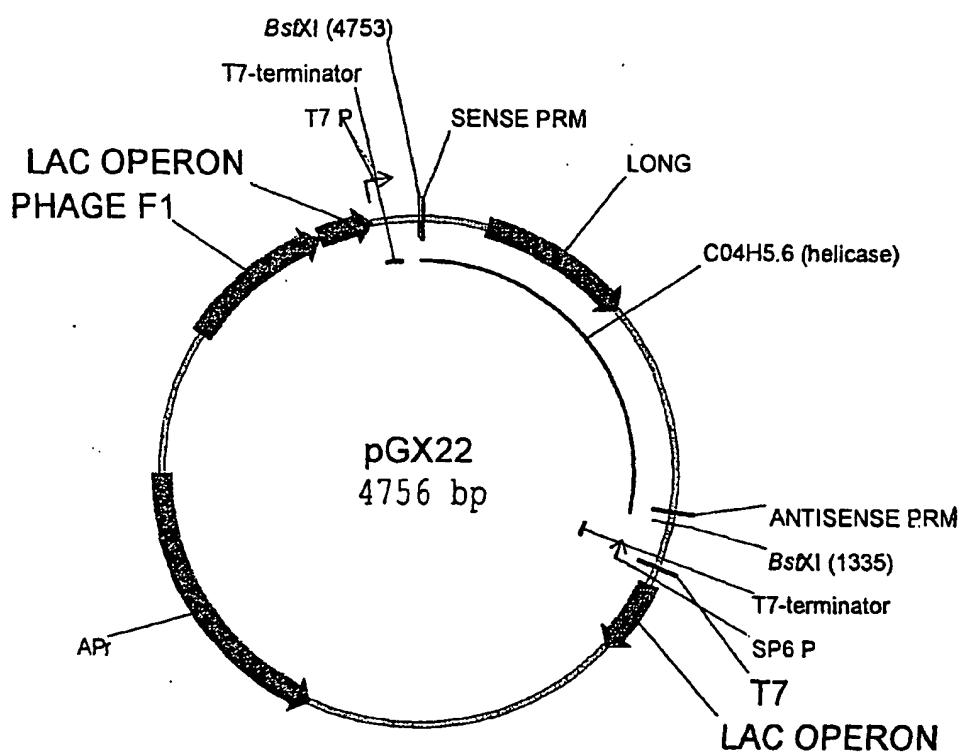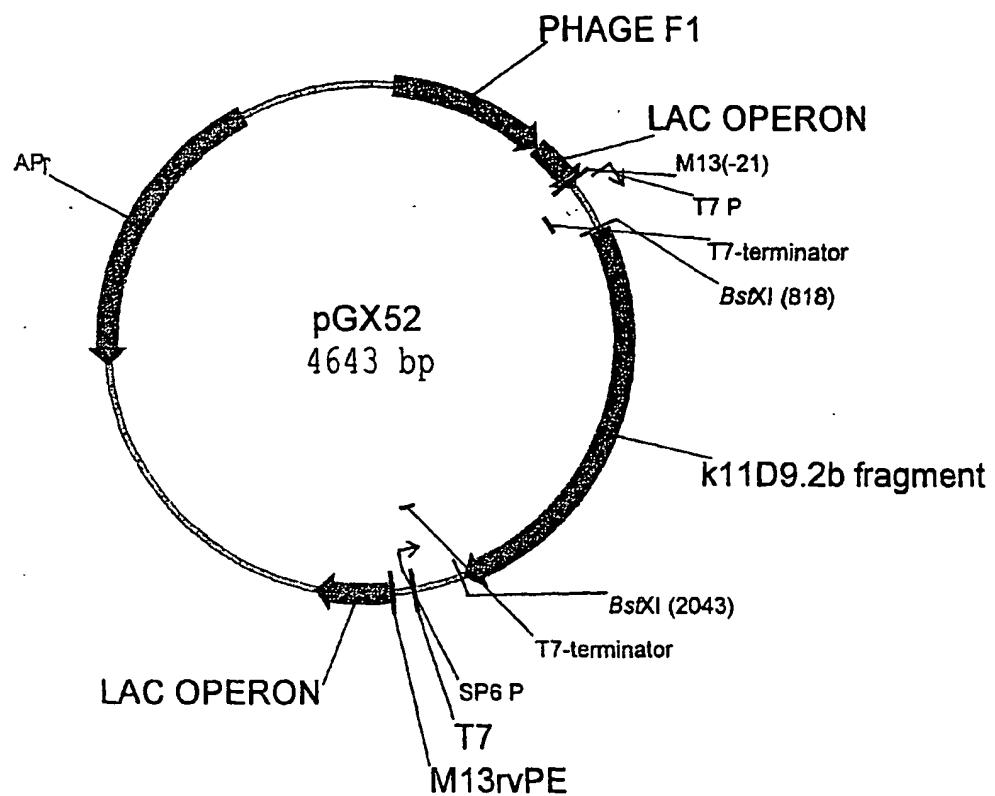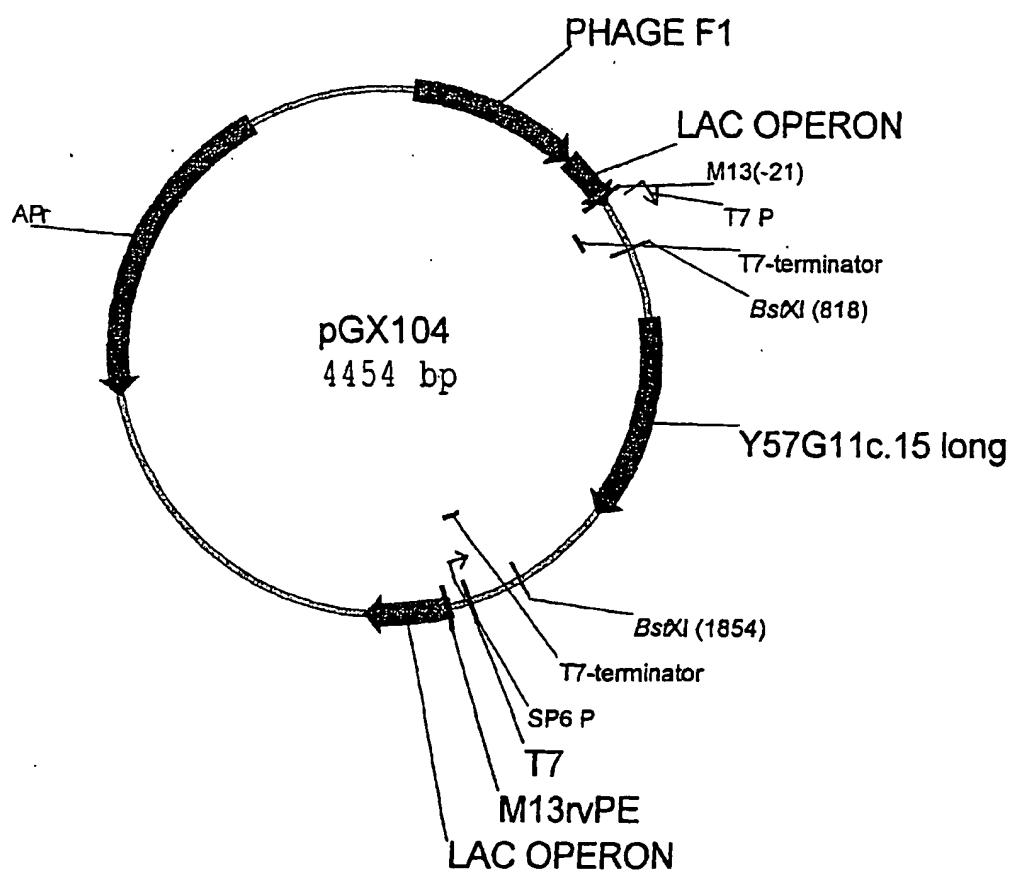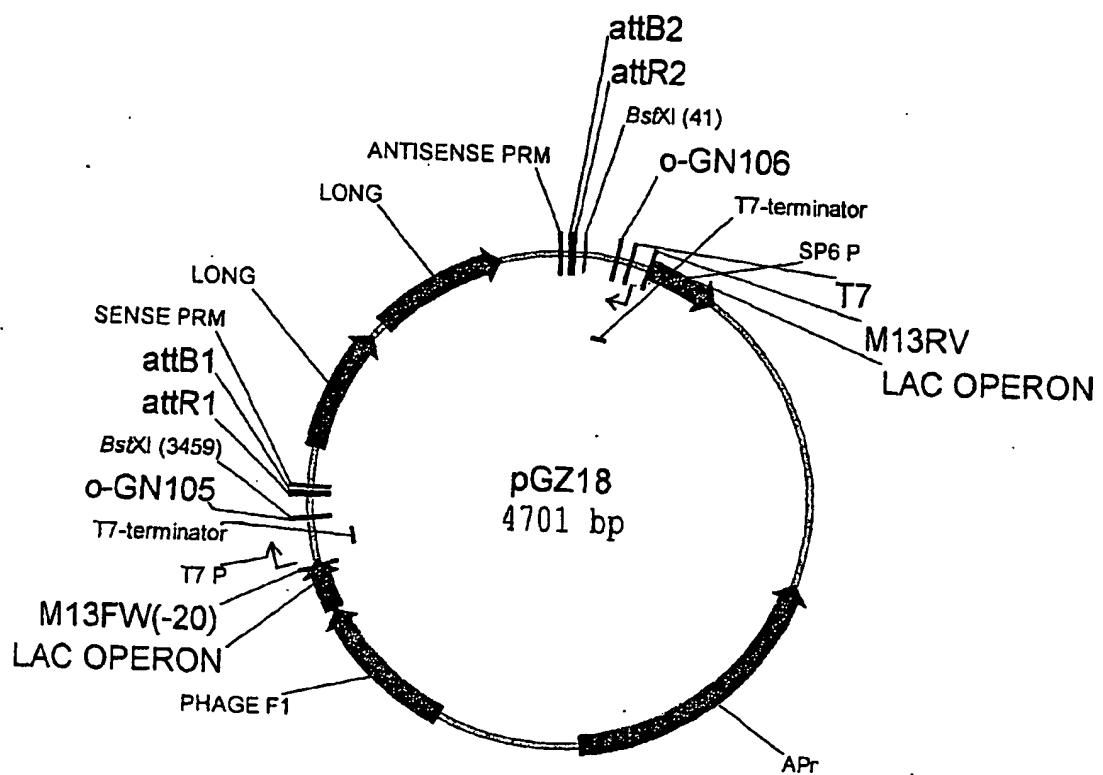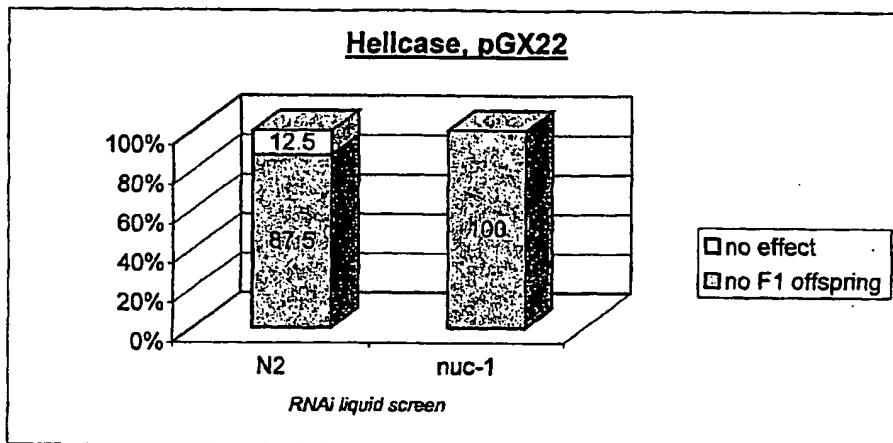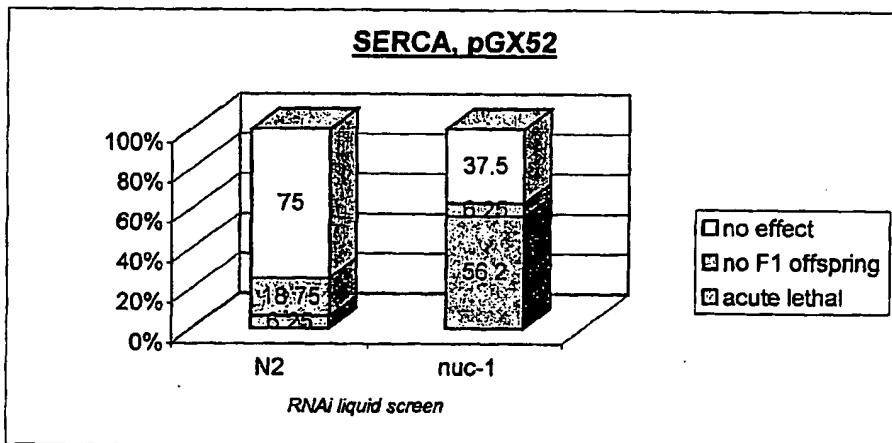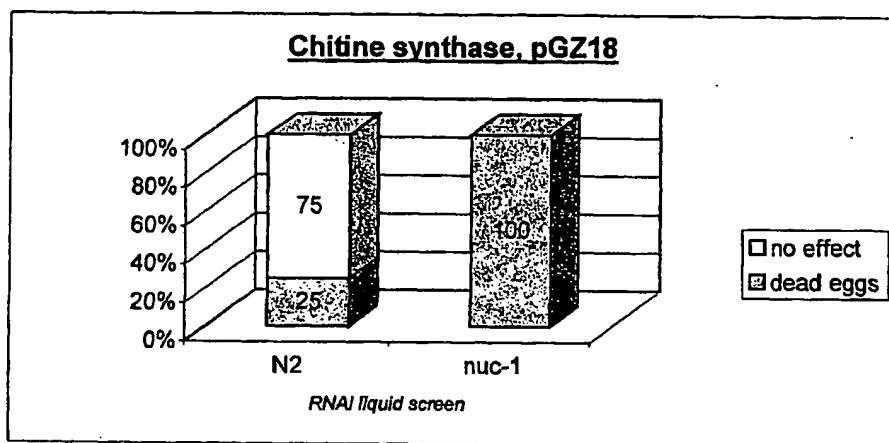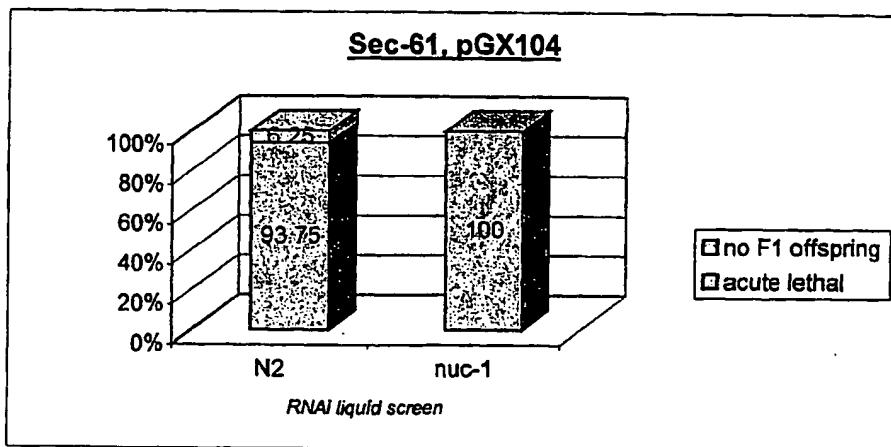
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<210> 4
<211> 4643
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Plasmid pGX52

<400> 4
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6

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<210> 5
<211> 4454
<212> DNA

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Plasmid pGX104

<400> 5

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8

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<210> 6
<211> 4701
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Plasmid pGZ18

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